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Mitochondria sense with different kinetics the calcium entering into HeLa cells through calcium channels CALHM1 and mutated P86L-CALHM1

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ABSTRACT

The novel Ca^{2+} channel CALHM1 (Calcium Homeostasis Modulator 1) generates cytosolic Ca^{2+} transients ($[Ca^{2+}]_c$) that regulate the production of amyloid beta (Aβ). Its mutated channel P86L-CALHM1 has been associated to Alzheimer's disease (AD). Using cytosolic- and mitochondrial-targeted aequorins, we have investigated here whether mitochondria sense with similar or different kinetics the Ca^{2+} entering into Hela cells and the Ca^{2+} released from the endoplasmic reticulum (ER), in control and in cells transfected with CALHM1 and P86L-CALHM1. We have shown that mitochondria sense Ca^{2+} entry in the three cell types; however, the $[Ca^{2+}]_c$ and mitochondrial Ca^{2+} transients $[Ca^{2+}]_m$ had substantially slower kinetics in cells expressing P86L-CALHM1. Mitochondria also sensed the ER Ca^{2+} released by histamine, but in CALHM1 and P86L-CALHM1 cells the kinetics was faster than that of control cells. Data are compatible with the idea that mutated CALHM1 may cause mitochondrial Ca^{2+} overload, suggesting how these cells may become more vulnerable to apoptotic stimuli.

Introduction

Abnormal cellular calcium (Ca^{2+}) homeostasis has been implicated in various neurodegenerative diseases including Alzheimer's Disease (AD) [1]. This is so because Ca^{2+} plays a critical role in the health of the cell, such that mild elevation of the cytosolic concentration of free Ca^{2+} ions ($[Ca^{2+}]_c$) promotes neuronal survival and plasticity, while higher $[Ca^{2+}]_c$ elevations can cause neurotoxicity [2–4]. Thus, alterations of Ca^{2+} homeostatic mechanisms associated with ageing, mutations in amyloid precursor protein (APP) and presenilins, or a dysfunction of Ca^{2+} fluxes at the ER, all lead to neurotoxicity [5].

How Ca^{2+} dyshomeostasis leads to neuronal death is uncertain. Perturbation of Ca^{2+} fluxes augment the production of amyloid beta 42 (Aβ42) peptide [6] and causes greater neuronal vulnerability to excytotoxic stimuli and Aβ exposure [7,8]. In addition, Aβ augments the Ca^{2+} concentration at the mitochondrial matrix ($[Ca^{2+}]_m$) [9]. Furthermore, Aβ amplifies Ca^{2+}-induced mitochondrial damage [10] and a mutation of PINK1 (a gene associated with familial Parkinson's disease) results in alteration of mitochondrial Ca^{2+} cycling and increased vulnerability to Ca^{2+}-induced mitochondrial toxicity [11]. Thus, the view that mitochondrial Ca^{2+} overload initiates the metabolic cascade leading to neuronal death is widely accepted [12,13].

A recent study [14], showed that the P86L polymorphism of the novel calcium channel CALHM1 (Calcium Homeostasis Modulator 1) is significantly associated with AD [15] although other studies failed to find such association [16]. Expression of CALHM1 was found in all brain regions and cells of neuronal linage, and is located at the ER and the plasmalemma. The channel appears to be constitutively open as membrane depolarization is not required for Ca^{2+} influx, is insensitive to classical blockers of voltage-gated calcium channels but is inhibited by nonspecific cobalt. CALHM1 generated Ca^{2+}-selective cation currents at the plasma membrane. The transient expression of P86L-CALHM1 mutated channel promotes Aβ accumulation by altering membrane Ca^{2+} permeability and $[Ca^{2+}]_c$ [14].

In the light of the role of mitochondrial Ca^{2+} movements in oxidative stress and neuronal death [17,18], we felt interesting to explore whether the Ca^{2+} transients generated by CALHM1 and P86L-CALHM1 were sensed by mitochondria in a similar or different fashion. To answer this question we transfected CALHM1 and P86L-CALHM1 in HeLa cells and studied the kinetics of the $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ transients generated by Ca^{2+} reintroduction. By following those Ca^{2+} changes using cytosolic- and mitochondrial- targeted aequorins, we discovered that both channels generated $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ transients; however, P86L-CALHM1-induced Ca^{2+} transients were

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substantially slower than those generated by CALHM1. This difference may be relevant in the context of the mitochondrial Ca^{2+} overload, neuronal cell death and proposed association between the P86L mutation of CALHM1 and AD [14].

Materials and methods

HeLa cells source, culture, and transfection. HeLa cells were grown in plastic flasks in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 25 U/ml penicillin and 25 μg/ml streptomycin. The experiments were performed with cells seeded on 13-mm diameter coverlips, in 24-well plates and grown to 60–70% confluence after 24 h in the incubator at 37 °C and 5% CO_2. Transfection with the genetically encoded photoprotein aequorin, targeted to the cytosol (cyt_AEQ) or targeted to mitochondria (mit_AEQ) was achieved by using Metafectene [19]. CALHM1 or P86L-CALHM1 were transiently co-transfected with aequorins, in a ratio 1:1, by using Metafectene. Experiments to measure $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ changes were performed 36–48 h after transfection. The two recombinant proteins were expressed in the same subset of cells, as shown by [20].

Measurements of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ with aequorin. HeLa cells expressing cyt_AEQ or mit_AEQ were reconstituted by adding 5 μM wild type coelenterazine for 1 h before the experiment. The cell monolayer was continuously superfused with Krebs–Hepes buffer (KHB) of the following composition (mM): 125 NaCl, 5 KCl, 1 Na_3PO_4, 1 $MgSO_4$, 5.5 glucose, 20 HEPES, with pH 7.4 and at room temperature (24 ± 2 °C); the zero Ca^{2+} solution contained 0.5 mM EGTA (ethylene glycol tetraacetic acid). To induce calcium entry, KHB deprived of Ca^{2+} was changed by another solution containing 1 mM $CaCl_2$, as specified in figure legends. Light emission was measured in a purpose-built luminometer and calibrated in terms of $[Ca^{2+}]$, as described by [21]. At the end of the experiment, cells were lysed by superfusing them with KHB containing 10 mM $CaCl_2$ and 100 μM digitonin, in order to expose cells to excess Ca^{2+} to burn out the aequorin remaining at the end of each experiment.

Chemicals. Wild type coelenterazine was from Labnet Biotecnica (Madrid, Spain). Metafectene was from Biontex, Munich, Germany. Histamine was purchased from Sigma (Madrid, Spain). The cDNA encoding for aequorins were a generous gift of Prof. Tullio Pozzan. The cDNA encoding for CALHM1 and P86L-CALHM1 were a generous gift of Prof. Philippe Marambaud (The Albert Einstein College of Medicine, Bronx, 10461, New York, USA) and (Litwin-Zucker Research Centre for the Study of AD, The Einstein Institute for Medical Research, North Shore-LIJ, Mantrasset, NY, 11030, USA).

Statistics. Values are given as means ± SE. The statistical differences between means were assessed by ANOVA test, when required. Differences between experimental groups were established as significant with a p value smaller than 0.05.

Results and discussion

To study the $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ transients in control HeLa cells (transfected with an empty plasmid) and in cells co-transfected with aequorins, CALHM1 and P86L-CALHM1, we followed the procedure of [14]. We first perifused cells with a $0Ca^{2+}$/EGTA solution for 2 min and subsequently, this solution was changed by another containing 1 mM Ca^{2+} (no EGTA).

Fig. 1A shows three superimposed example traces on the $[Ca^{2+}]_c$ variations elicited by Ca^{2+} reintroduction. In control cells, the $[Ca^{2+}]_c$ rapidly rose to a peak of 3.414 μM and then quickly decayed to reach baseline levels in about 20 s. This $[Ca^{2+}]_c$ transient was surely produced by Ca^{2+} entering cells through store-operated calcium channels (SOCCs) that open by ER Ca^{2+} store depletion taking place during cell perifusion with the $0Ca^{2+}$/EGTA solution [22]. The rapid decay of the $[Ca^{2+}]_c$ elevation was likely due to rapid Ca^{2+} clearance secondary to SOCC closing upon refilling of the ER Ca^{2+} store, and to rapid clearance by mitochondria through the Ca^{2+} uniporter [23].

The $[Ca^{2+}]$ peak of CALHM1 cells was substantially higher although the rise rate overlapped that of control cells (Fig. 1A). The easiest interpretation for this observation is the contribution of SOCCs as in control cells, and additional Ca^{2+} entry through CALHM1 channels inserted into the plasmalemma, as happened to be the case in HT-22 cells transfected with CALHM1 [14]. The slower decay of the $[Ca^{2+}]_c$ elevation could be explained by two

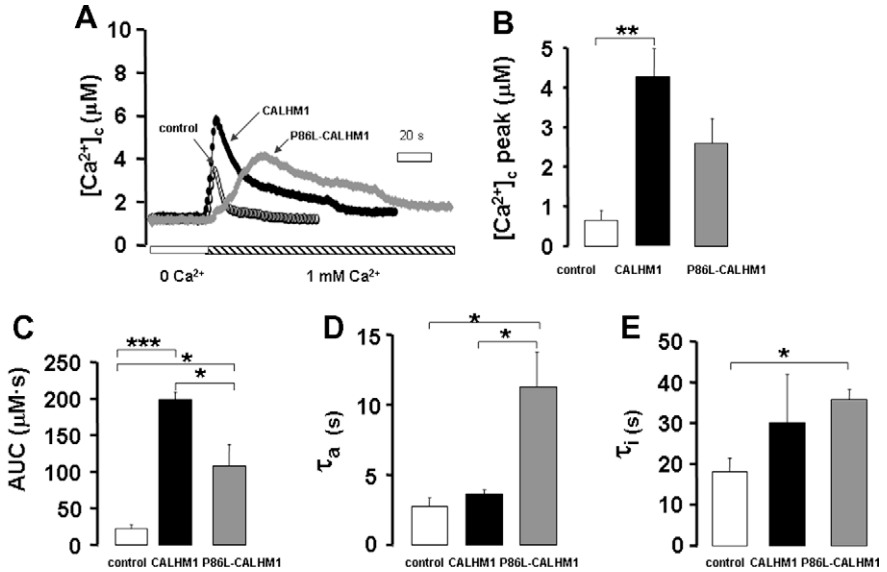

Fig. 1. Different kinetics of the cytosolic Ca^{2+} transients ($[Ca^{2+}]_c$) generated by Ca^{2+} reintroduction in control HeLa cells, and in cells transfected with CALHM1 or P86L-CALHM1, measured with cyt_AEQ. (A) Example traces of the time course of $[Ca^{2+}]_c$ elevations (ordinate) elicited during the time period of Ca^{2+} reintroduction indicated by the bottom horizontal bar. Panels B, C, D and E indicate, respectively, peak $[Ca^{2+}]_c$ transient amplitude, area under the curve (AUC), and time constants for the activation (τ_a) and inactivation (τ_i) of the transients. Bar graphs of panels B–E were constructed with pooled data from 15 experiments made with cells from at least 3 different cell cultures, performed with protocols as those of panel A. Data are means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

mechanisms: (i) lesser Ca^{2+}-dependent inactivation of CALHM1 channels, compared with SOCCs; and (ii) the higher $[Ca^{2+}]_c$ peak will produce a greater $[Ca^{2+}]_m$ in such a manner that the subsequent Ca^{2+} release trough the mitochondrial Na^+/Ca^{2+} exchanger [24,25] will contribute to the longer high $[Ca^{2+}]_c$ levels.

Still more different was the time course of the $[Ca^{2+}]_c$ elevation produced by Ca^{2+} reintroduction into P86L-CALHM1 cells. The rise rate was markedly slowed down and a peak $[Ca^{2+}]_c$ was reached only after a 25.86-s delay. In fact such peak turned on into a slowly decaying plateau. Furthermore, the $[Ca^{2+}]_c$ did not reach baseline levels even after the 2-min recording period. This could be interpreted by slow inactivation of P86L-CALHM1 channels and by greater mitochondrial Ca^{2+} uptake and slower mitochondrial Ca^{2+} release into the cytosol.

Pooled data from 15 experiments made with each cell type are shown in the bar graphs of Fig. 1. Thus, the $[Ca^{2+}]_c$ peaks in CALHM1 and P86L-CALHM1 were 6- and 4-fold higher, compared with control cells (Fig. 1B). The area under the curve (AUC), an indication of total Ca^{2+} entry and its redistribution into organelles was 9.08- and 4.95-fold higher in CALHM1 and P86L-CALHM1 cells, compared with control cells. (Fig. 1C). On the other hand, the time constant for activation of $[Ca^{2+}]_c$ transients (τ_a) were similar in control and CALHM1 cells; however, τ_a was 4-fold higher in P86L-CALHM1 cells, indicating a much slower rate of $[Ca^{2+}]_c$ rise (Fig 1D). Finally, the decay rate of $[Ca^{2+}]_c$ elevations was about 2-fold higher in CALHM1 and P86L-CALHM1 cells, compared with control cells.

In summary, Ca^{2+} reintroduction produced $[Ca^{2+}]_c$ elevations in control HeLa cells as well as in cells transfected with CALHM1 or P86L-CALHM1 cDNAs. However, such $[Ca^{2+}]_c$ elevations were considerably greater and exhibited a slower decay in CALHM1 cells, compared with control cells. Furthermore, P86L-CALHM1 cells generated a smaller $[Ca^{2+}]_c$ elevation with slower activation and inactivation kinetics.

Mitochondria are sensing the $[Ca^{2+}]_c$ elevations elicited by Ca^{2+} entry through voltage-dependent calcium channels [26] and SOCCs [27], as well as those elicited by Ca^{2+} release from the ER store [25]. It was therefore, of interest to know whether mitochondria were also sensing the $[Ca^{2+}]_c$ elevations described above. To this aim we transfected HeLa cells with a mitochondrial-targeted aequorin and the cDNAs for CALHM1 and P86L-CALHM1, and performed experiments with Ca^{2+} reintroduction following a protocol similar to that used for measuring $[Ca^{2+}]_c$ changes. It was anticipated that the changes of $[Ca^{2+}]_m$ would somehow parallel those seen with cytosolic Ca^{2+} [28].

Fig. 2A shows three superimposed traces of the time course of $[Ca^{2+}]_m$ elevations triggered by Ca^{2+} reintroduction. In control cells $[Ca^{2+}]_m$ rose to a peak of 2.4 μM that was followed by a decay to reach baseline in a about a minute. In CALHM1 cells the $[Ca^{2+}]_m$ peak reached 7.7 μM and decayed with a slower time course; at the end of the 2-min recording peak, the $[Ca^{2+}]_m$ did not reach baseline levels. Finally the example trace obtained from P86L-CALHM1 cells had a quite distinct shape; $[Ca^{2+}]_m$ was slowly increasing to reach a peak of 3.9 μM after 23-s; this peak was followed by a plateau and later on by a slow decay phase, but $[Ca^{2+}]_m$ did not reach baseline levels after the 2-min recording period. Thus, the morphology of the $[Ca^{2+}]_m$ traces are like a mirror image of the $[Ca^{2+}]_c$ changes, indicating that mitochondria were sensing those changes in the three cell types. Because of the low Ca^{2+} affinity of the mitochondrial uniporter (mUP), it is plausible that mitochondria sensing the Ca^{2+} that enters through CALHM1 and P86L-CALHM1 are those located at subplasmalemmal sites [26].

Pooled data of 15 experiments performed with each cell type are shown in the bar graphs of Fig. 2. Note first that the $[Ca^{2+}]_m$ peak in CALHM1 cells was 4-fold higher than in control cells. In contrast, this peak was only 1.8-fold higher in P86L-CALHM1 cells (Fig. 2B). Also, the AUC was 6-fold higher in CALHM1 cells, as compared with control cells. It was interesting that the AUC of P86L-CALHM1 cells was 4.5-fold higher than in control cells. Thus, compared with the peak, the area of the $[Ca^{2+}]_m$ curve was closer in cells expressing the wild type and the mutated channel (compare panels B and C in Fig. 2). The τ_a for the $[Ca^{2+}]_m$ increase was 3.5-s in control and CALHM1 cells; this value was almost twice as high in the case of P86L-CALHM1 cells (Fig. 2D). Finally, the τ was around 18-s in control and CALHM1 cells, and 38-s in P86L-CALHM1 cells, indicating a slower Ca^{2+} rise and decay in mitochondria of cells transfected with the mutated channel.

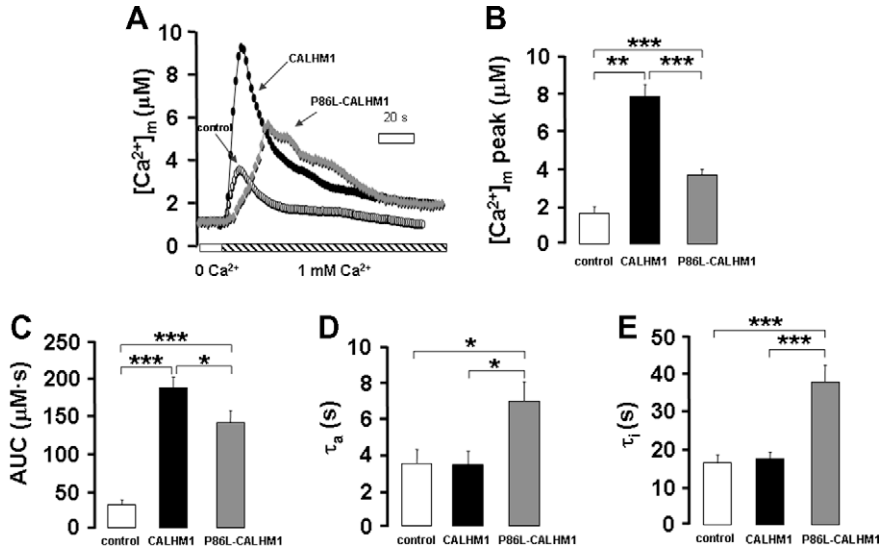

Fig. 2. Different kinetics of the mitochondrial Ca^{2+} transients ($[Ca^{2+}]_m$) generated by Ca^{2+} reintroduction in control HeLa cells, and in cells transfected with CALHM1 or P86L-CALHM1, measured with mit_AEQ. (A) Example traces of the time course of $[Ca^{2+}]_m$ elevations (ordinate) elicited during the time period of Ca^{2+} reintroduction indicated by the bottom horizontal bar. Panels B, C, D and E indicate, respectively, peak $[Ca^{2+}]_m$ transient, area under the curve (AUC), and time constants for the activation (τ_a) and inactivation (τ_i) of the transients. Bar graphs of panels B–E were constructed with pooled data from 15 experiments made with cells from at least 3 different cell cultures, performed with protocols as those of panel A. Data are means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

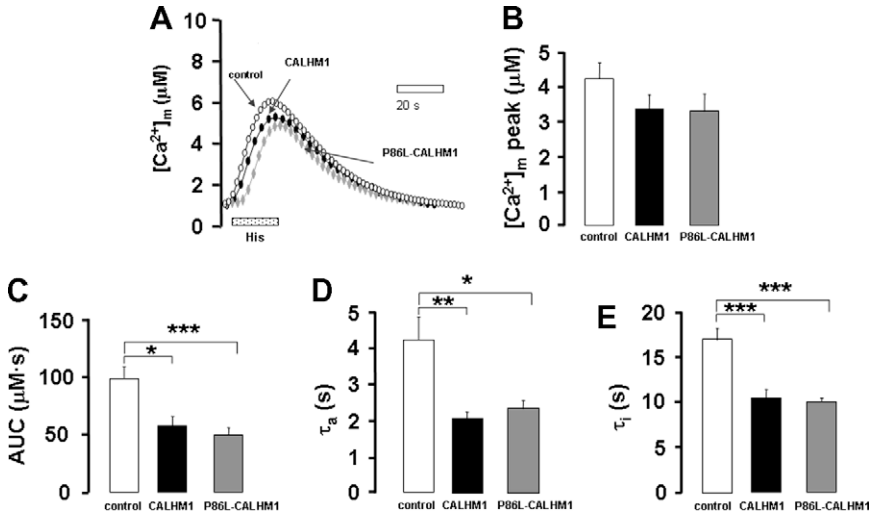

Fig. 3. Different kinetics of the mitochondrial Ca^{2+} transients ($[Ca^{2+}]_m$) generated by histamine stimulation of control HeLa cells, and cells transfected with CALHM1 or P86L-CALHM1, measured with mit_AEQ. (A) Example traces of the time course of $[Ca^{2+}]_m$ elevations elicited by histamine, given as indicated by the bottom horizontal bar. Panels B, C, D and E indicate, respectively, peak $[Ca^{2+}]_m$ transient, area under the curve (AUC), and time constants for the activation (τ_a) and inactivation (τ_i) of the transients. Bar graphs of panels B–E were constructed with pooled data from 15 experiments made with cells from at least 3 different cell cultures, performed with protocols as those of panel A. Data are means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In summary, mitochondria are sensing the $[Ca^{2+}]_c$ changes elicited by Ca^{2+} reintroduction in the three cell types studied. However, in cells transfected with CALHM1 and P86L-CALHM1, mitochondria took up 4- to 6-fold greater amounts of Ca^{2+}, as compared with control cells. Of note, the time course of $[Ca^{2+}]_m$ changes was substantially slower in P86L-CALHM1 cells, compared with cells transfected with CALHM1; nevertheless, the total amount of Ca^{2+} seen by mitochondria was similar in both cell types.

So far, we have seen that mitochondria sense the Ca^{2+} entering cells trough plasmalemmal calcium channels. Because mitochondria also sense Ca^{2+} released by the ER store [28], we considered of interest to investigate the kinetics of $[Ca^{2+}]_m$ changes upon stimulations of ER Ca^{2+} release. Cell challenging with 100 μM histamine to trigger inositol triphosphate receptors (IP_3R) mediated ER Ca^{2+} release [28], produced a rapid increase of $[Ca^{2+}]_m$ (Fig 3A). The averaged peak $[Ca^{2+}]_m$ was 4.6 μM in control cells; in CALHM1 and P86L-CLAHM1 cells, peaks were around 3.5 μM (Fig. 3A). More significant differences were found in the total Ca^{2+} taken up by mitochondria. Thus, AUC amounted to 100 μM·s in control cells; this figure was reduced by half in CALHM1 and P86L-CALHM1 cells (Fig. 3C). On the other hand, τ_a was also halved in cells transfected with the wild type or the mutated channel compared with control cells (Fig. 3D). Finally, the τ_i was also reduced in transfected cells, compared with control cells (Fig. 3E).

It was interesting that the kinetics of $[Ca^{2+}]_m$ elevations followed a similar pattern when Ca^{2+} offered to mitochondria was coming from the ER store in both cell types. This considerably differed from the kinetics of $[Ca^{2+}]_m$ elevations when Ca^{2+} came from the extracellular milieu, that was much slower in P86L-CALHM1 cells, compared with CALHM1 cells (compare Fig. 2D and E with 3D and E). It was also of interest that AUC was reduced in transfected cells, compared to control cells. This may be explained by the fact that CALHM1 is also expressed in the ER membrane [14]; it was therefore, likely that this elicited ER Ca^{2+} leak and a store with smaller Ca^{2+} concentrations. If so, histamine will release lesser Ca^{2+} into the cytosol and hence mitochondria will take up smaller amounts of Ca^{2+}.

Our data may be relevant for understanding the role of Ca^{2+} in the process of neuronal cell death occurring in AD. Various factors including high $[Ca^{2+}]_m$ and mitochondrial depolarization, trigger the opening of the mitochondrial permeability transition pore (mPTP) that, if prolonged, may favour the release of apoptotic factors and cell death [24]. In fact, mutation of PINK1 slows down the release of Ca^{2+} through the mitochondrial Na^+/Ca^{2+} exchanger (mNCX), and augments Ca^{2+}-induced mitochondrial and cell toxicity [11]. This is likely due to mitochondrial Ca^{2+} overload due to Ca^{2+} release, a situation similar to the slow $[Ca^{2+}]_m$ transients secondary to Ca^{2+} entry stimulation in P86L-CALHM1 cells that we have observed in the present experiments (Fig. 2). P86L-CALHM1 augments the cell production of Aβ [14], and Aβ augments $[Ca^{2+}]_m$ [9] and amplifies Ca^{2+}-induced mitochondrial damage [10].

Conclusion

In conclusion, we have shown that HeLa cells transfected with P86L-CALHM1 channel exhibit a slower mitochondrial Ca^{2+} uptake and release, compared with control cells and with cells transfected with CALHM1 channel. Since the mutated channel has been associated to AD, we suggest that AD patients carrying the P86L mutation of CALHM1 channel may have neuronal mitochondria more vulnerable to Ca^{2+} overload and to apoptotic stimuli.

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